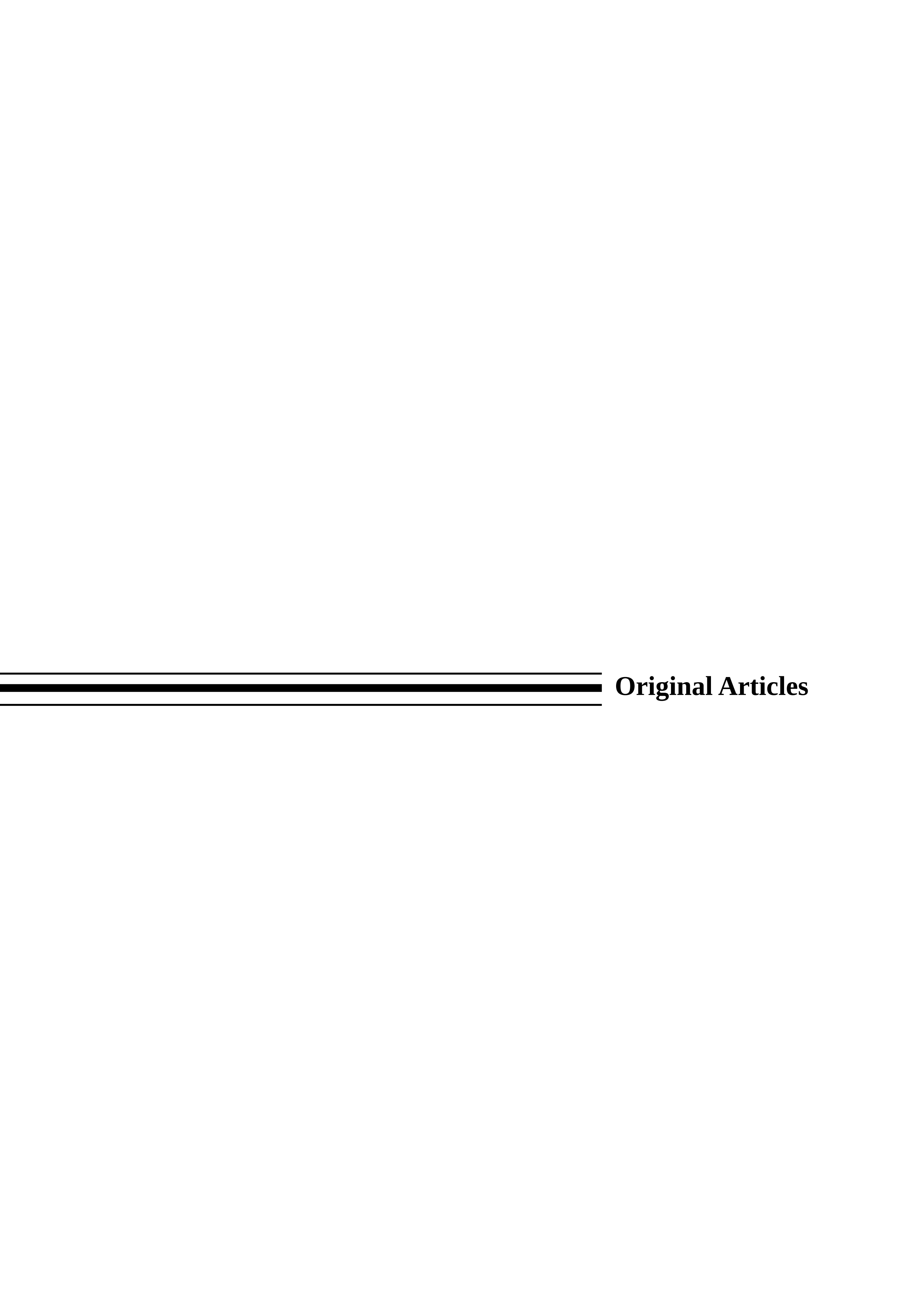


Original Articles

An Ideal Selective Anti-Cancer Agent *In Vitro:* I – Tissue Culture Study of Human Lung Cancer Cells A549

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ABSTRACT. Management of cancer is one of the challenging problems in medical practice as there are no available medical modalities that can selectively kill cancer cells without adverse effect on normal living cells or the functions of vital organs. Tissue culture of human lung cancer cells (A549) was used in studying the effect of agent, PM 701, to test its effect on the behavior of the cancer cells as compared with that of normal cells (human skin fibroblasts). This new agent proved to be effective in killing lung cancer cells through its effect on the nuclei, limiting the division of these cells, causing degeneration and apoptosis. Conversely, PM 701 exhibited nourishing effects on normal cultured skin fibroblasts; this implies that this agent may have selective cancer cell killing effect and reparative effect on normal dividing cells (fibroblasts). The results showed that all tested concentrations of PM 701 inhibited the growth of the human lung cancer cells (A549 cells), with maximum effect at medium concentration. There is immediate lethal effect of this agent on cancer cells noticed at the first 10-minutes in live experiment. The present study represents the first experience in using PM 701 as a selective anticancer agent at tissue culture level.

Keywords: Lung cancer cells, A549, Tissue culture, Anticancer agent, Fibroblasts.

Introduction

Lung cancer is considered to be an aggressive disease that affects a large number of patients yearly. The patients are more likely to present with extensive

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disease and have poor prognosis even when treated promptly after diagnosis. It is considered to be one of the leading causes of cancer related deaths worldwide. It kills roughly thousands of patients every year, largely because it is usually diagnosed late. The global incidence of lung cancer is 1,240,000 (*i.e.*, 901,746 new cases in men, and 337,115 cases in women worldwide in 2001). In 2003, the incidence was reported to be 170,000 cases per year in United States[1-7].

It is of primary importance to find an anticancer agent that kills cancer cells without unacceptable toxicity to patient's own tissues and or functions. A correlation between apoptosis and therapeutic response indicated that apoptosis, indeed, is a goal of cancer therapy[8-12]. Although induction of apoptosis (cell death mediated by caspases) determines responses to cancer therapy, this approach is limited by lack of selectivity in the available apoptosis-inducing agents[8]. Furthermore, most cancers, almost by definition, are resistant to apoptosis, growth arrest and cell senescence. Therefore, the rational of drug combination (at mechanism-based doses and sequences) aimed at matching targets of selective cancer cell killing (apoptosis) with normal cells preservation. All new therapies aimed to ensure this selectivity, by combining an apoptosis and senescence – inducing agents with the inhibitors of apoptosis at the same time to protect normal cells as tissue-selective therapy[8].

The best approach to evaluate the effect of new materials should be *in vitro* by utilizing the growing mammalian cells at tissue culture level and not on the living organism[13-17]. The later was applied in our laboratory at the Tissue Culture Unit (TCU), King Fahd Medical Research Center (KFMRC). Where PM 701 was tested as an anticancer agent on the cultured lung cancer cells (A549) and at the same time its effect tested was on the cultured normal skin fibroblast.

We are presenting the first experience in using this alternative agent at the tissue culture level and confirmed with documented evidence the selective anticancer effect of PM 701, in causing programmed cancer cell death (apoptosis) with novel flourishing effect on the normal cells.

Materials and Methods

Media:

The following commercially available media were prepared according to published literature, these include:

Ordinary media, minimal essential medium (MEM) is a rich, multipurpose medium that was used for cultivation of mammalian cells[18].

Phosphate-buffered-saline (PBS) is a phosphate-buffered physiological saline solution that is calcium- and magnesium-free solution[17, 18].

Trypsin: [17, 18].

Examined media:
The examined agent which is a natural substance, easily available, cheap, sterile, and non-toxic according to chemical and microbiological testing. This agent was coined with name of (PM 701) and used by adding it to the ordinary media with different concentrations ranging from 1:10 to 1:100,000:

1 ml substrate: 10 ml media, which is called – 1 (high)
1 ml substrate: 100 ml media, which is called – 2
1 ml substrate: 1,000 ml media, which is called – 3 (mid)
1 ml substrate: 10,000 ml media, which is called – 4
1 ml substrate: 100,000 ml media, which is called – 5 (low)

Cell lines:
Human Lung Cancer Cells line
Human Lung Cancer Cells, non-small cell carcinoma (A549) was obtained from cell strain from American Type Cultural Collection (ATCC), available in the cell bank of Tissue Culture Unit.

Human Skin Fibroblast Cells

The normal human specimens (human skin fibroblasts) were obtained from King Abdulaziz University Hospital (KAUH), after circumcision operations. The specimens transported immediately within 5 min after excision in previously prepared bottles of MEM media. The human skin samples (circumcision of penile foreskin) were cut into small fragments, minced, and gently agitated in Trypsin solution (GIBCO) at a concentration of 0. 25%, 0.1% glucose, and 0.02% EDTA for 15 min. Trypsin action was quenched by MEM when intercellular separation was observed. The supernatant suspension containing the dissociated cells was removed and centrifuged at 100 xg for 10 min, cells were re-suspended in MEM containing 20% fetal calf serum (F.C.S.; ICN) heat inactivated (56°C for 30 min). Cells were adjusted to 1 × 105 cells/ml and plated into tissue culture flask 25 cm^2 then incubated in a humidified incubator in an atmosphere of 5% CO_2 at 37°C.

Cells were grown in 25 cm^2 polystyrene flasks and passage biweekly. Cells suspended in PBS, centrifuged, re-suspended in culture medium MEM as described previously[18].

Tissue Culture Experiments

Each group of cells was cultured in Petri dishes using control and examined media for normal and cancer cells. The results from abnormal tissues compared with structure of normal tissues.

Three types of experiments were done in this project:

1 – Fixing and Staining cells: Each group of cells were plated onto Petri dishes in MEM media for 24 h, then the media changed with examined media (with different concentrations) and control media and incubated at 37°C for 24 h or 48 h or 72 h or 96 h or 120 h.

Each group of cells fixed in 4% formaldehyde for 5 min at room temperature after double washing with 1 × PBS each for 5 min.

Then cells stained with Coomassie blue for 5-10 min followed by repeated washing with tap water[17, 18].

2 – Imaging cells by time-lapse in live experiment: cancer and normal cells were allowed to grow in ordinary media for 24 h. Cells were imaged as a control for 5-10 min at controlled conditions in live experiment, the normal media then changed with examined media during time-lapse images of living cells[17], for one and a half hour.

Live images typically were recorded using a CCD camera and saved in PC computer. Images were processed using computer programs. The data then analyzed using statistics computer programs.

3 – Cells counting experiments:

This can be performed using a hemocytometer. Trypan blue was used as a dye for viable cell count; the number of cells that can exclude the stain (*i.e.*, have intact cell membrane) can be determined by counting the cells as described below:

1 – Cell suspension was prepared (1:1): 20 ml of cells with 20 ML of (0.4%) Trypan blue.
2 – With the cover slip is firmly in place, a drop of a cell suspension were transferred to both sides of the hemocytometer by carefully touching the edge of the cover slip with the pipette tip and allowing the chamber to fill by capillary action.
3 – All cells in four corner squares and Middle Square were counted and the average was calculated.
4 – Each large square of the hemocytometer, with cover–slip in place, represents a total volume of 0.1 mm^3 or 10-4 cm^3. Since 1 cm^3 is equivalent to approximately 1 ml the total number of cells per ml determined using the following calculation:

Cells/ml = the average of cells number (no. of cells / no. of squares) × 104
Total cells = cells / ml × the original volume of fluid from which the cell sample was removed.

Cell viability = total viable cells (unstained) / total cells × 104 × dilution factor (suspension cells: Trypan blue)[18].

Results

Tissue culture results: For this part of experiment, the effect of substance (PM 701) was examined on one type of cancer cells (non-small cell human lung cancer, A549). The results were compared with positive and negative control, where the substrate effect was examined on normal cells (human skin fibroblasts) and on cancer cells that incubated in ordinary media.

Although the results show that substrate (PM 701) destroys the cancer cells when adding to the incubated media, there is satisfactory evidence that the same substrate did not cause any harm to the normal cells.

The new media that contain the examined substrate attack the cancer cells when incubated in it, this was proved by the next three types of experiments that were given different parameter for the reaction of cells to the media.

1) ***Fixed and stained cells:*** This experiment shows the degree of injury and discomfiture of the cells to the incubated PM 701 medium. When we compare the cancer cells that incubated in ordinary media (MEM) for 24 h with the cancer cells that incubated in different concentration of examined substrate we noticed that the low concentration of PM 701 (–4 and –5) affect the growth of cells (Fig. 1) as the high concentration (–1), but the best effect appeared near the middle concentration (–3) (Fig. 2), whereas the effect on the cells includes the growth and reaction of cells and also the number of cells.

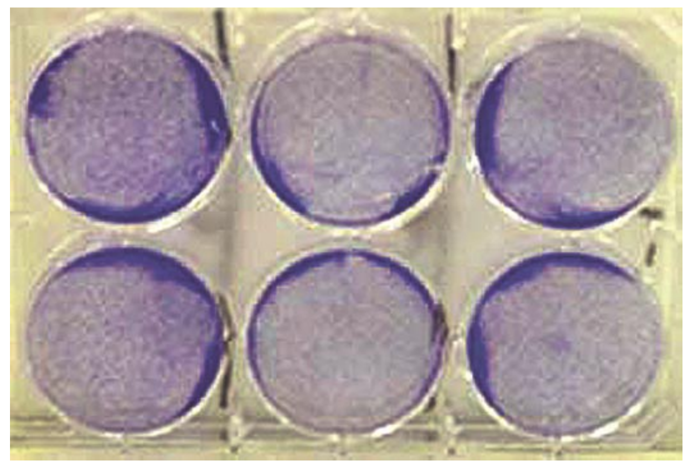


FIG. 1. Shows cancer cells incubated for 24 h, the first upper and lower dishes for the control in MEM media; the second upper and lower dishes the media with examined substrate (–5); the third upper and lower dishes the media with examined substrate (–4).

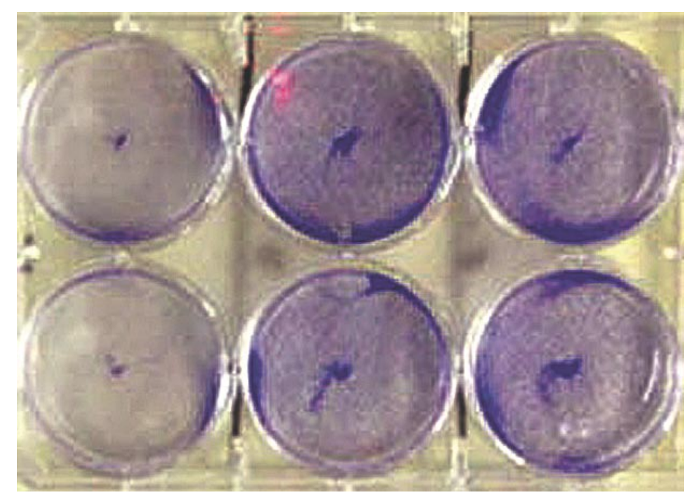


FIG. 2. Shows cancer cells incubated for 24 h, the first upper and lower dishes the media with examined substrate (–1), the second and third upper dishes the media with examined substrate (–2), the second and third lower dishes the media with examined substrate (–3).

The new media surrounded the cancer cells from all directions preventing them from direct reaction and contact with the surrounding environment; this indicates the isolation of cells from their surrounding. Furthermore, this substrate damaged the nuclei of the cancer cells.

The images of the fixed and stained cancer cells (A549) showed that they have been attacked, destroyed and decreased in number when incubated in media containing the examined substrate with different concentrations for 24 h (Fig. 3-5), also the cancer cells became very rare when grown in PM 701 for

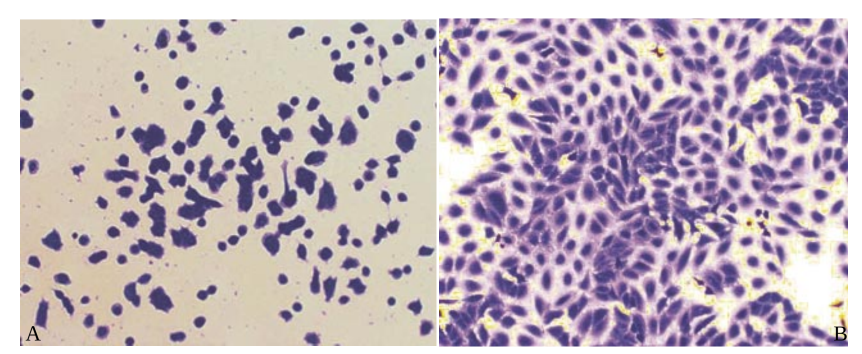


FIG. 3. Cancer cells A549 imaged (× 10) after incubation for 24 h, fixed and stained with Coomassie blue:
(A) in (–2) PM 701, note the decrease in the number of cells and the cells damage compared with the control cells that were incubated in MEM media (B).
Scale bars for all images 600 μm.

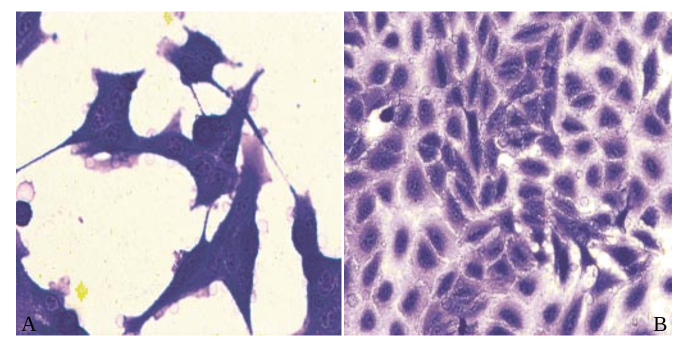


Fig. 4. Cancer cells A549 imaged (× 20) after incubation for 24 h, fixed and stained with Coomassie blue:
(A) in (–2) PM 701, note the damage of cells as compared with the control cells that were incubated in MEM media (B).

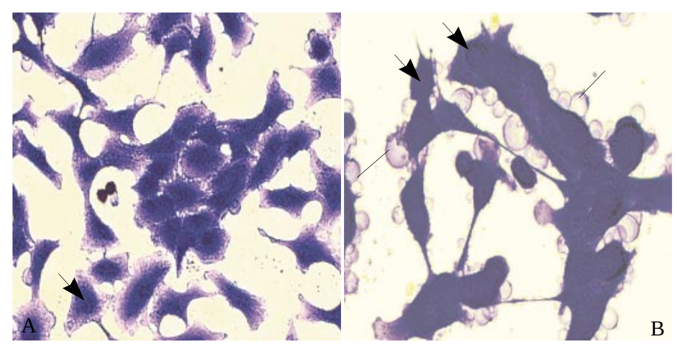


Fig. 5. Cancer cells A549 imaged after incubation for 24 h in (–3) PM 701:
(A) (× 20), (B) (× 40) note that the cells degenerated after surrounded by the new media (lines) and nuclei appeared to be damaged (arrows).

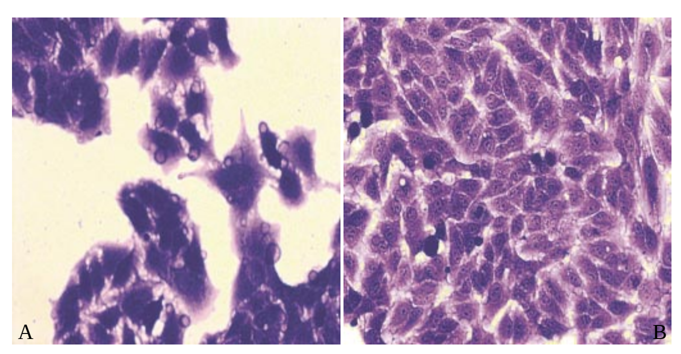


Fig. 6. Cancer cells A549 imaged after incubation in: (A) (–4 PM 705) for 120 h compared with control cancer cells incubated in (B) MEM for 120 h (× 40). It is well observed the high ability of cancer cells to divide, image (B).

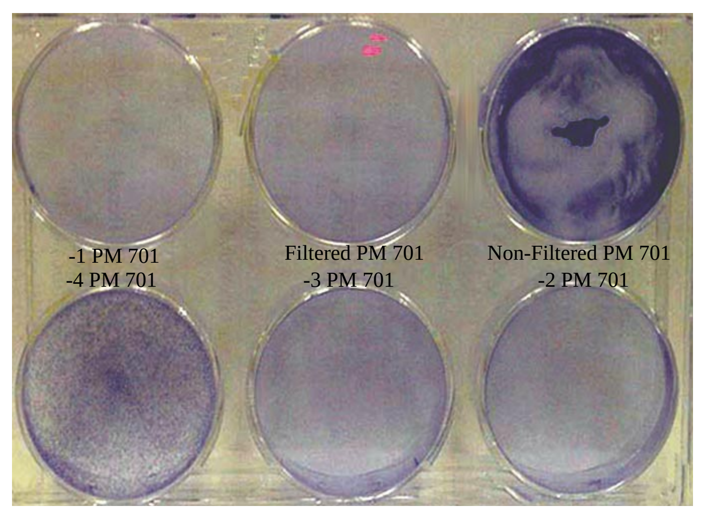


Fig. 7. Shows cancer cells incubated in PM 701 for 96 h: the upper first, second, and third dishes with subsequent conc. (–1, filtered and non-filtered substrates); the lower dishes for –4, –3, and –2 conc. of examined substrates.

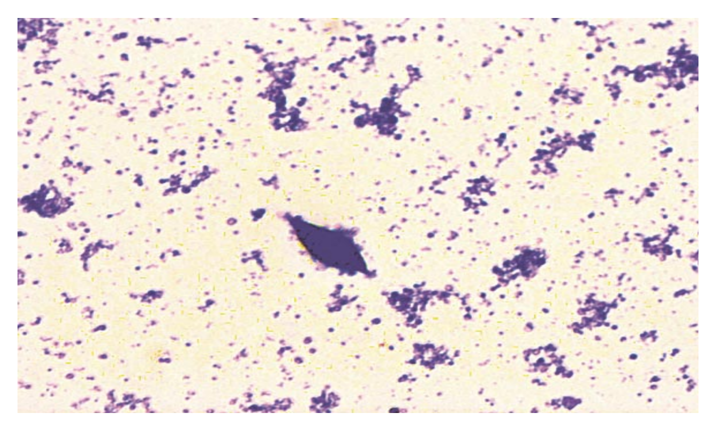


Fig. 8. Cancer cells A549 imaged after incubation in (–3 PM 705) for 96 h, note the loss of cells from the field (× 40).

long period (120 h) as shown (Fig. 6). The long period of incubation of cells in PM 701 for 96-120 h shows that cells destroyed completely with loss of cells from the field (Fig. 7, 8).

The images of fixed cells show that the examined substrate attacks the cancer cells preventing the growth and survival of these cells compared with cancer cells incubated in ordinary media, whereas the normal cells (human skin fibroblasts) appeared healthy with advanced growth and spreading of these cells (Fig. 9).

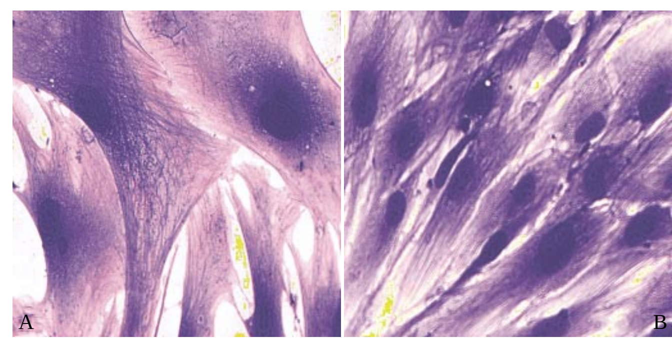


Fig. 9. Normal fibroblasts incubated in PM 701: (A) for 24 h; (B) for 96 h; notice the well spreading cells with obvious well developed cytoskeleton and organelles.

2) ***Live experiment:*** The experiment shows the direct effect of PM 701 medium on living cells. Cancer cells and normal fibroblasts allowed to grow in ordinary media for 24 h, cells imaged in this media as a control for 5-7 min (Fig. 10: Images 1a & 1b), then the media changed with the examined media under live conditions for cells imaging. This live experiment shows that the severe lethal effect of PM 701 on cancer cells started immediately after 5-6 min from adding the examined substrate (Fig. 10: Image 2b). The live observations of normal fibroblasts incubated in PM 701 as a control show that the fibroblast cell retracts after adding the new media immediately by pulling its pseudopodia, which might be explained by the introduction of the cell to this substrate. While the well spreading of the cell body of the fibroblasts, indicates the positive reaction between the cell and this new substrate (Fig. 10: Images 1a-4a). The cancer cells incubated in PM 701 showed that the substrate attacks the cell's nuclei, which is indicated by the appearance of pale ring around the nucleus. This leads to the degeneration of cells (Fig. 10: Images 3b and 4b; and Fig. 11: Images 5 and 6), which could not be avoided by re-growing the cells in ordinary media again. The severe effect on the nuclei of cancer cells limit the ability of cells to divide and survive, which indicates a high efficiency in killing cancer cells.

3) ***Cells count:*** This experiment gives the number of living cells or the viability of cells after incubated in PM 701. Results of cells count experiments show severe drop of cancer cells number when incubated in PM 701 compared with the number of control cells (cancer) that incubated in MEM media, (Fig. 12-15). Furthermore the normal cells (fibroblasts) number shows normal progress, when incubated in PM 701 with very little depression in the first stages of incubation (Fig. 15). The depression of fibroblasts number incubated in PM 701 in the beginning of the experiment explained by the introduction of cells to the new media. While the fixed images indicate that normal cells (fibro-

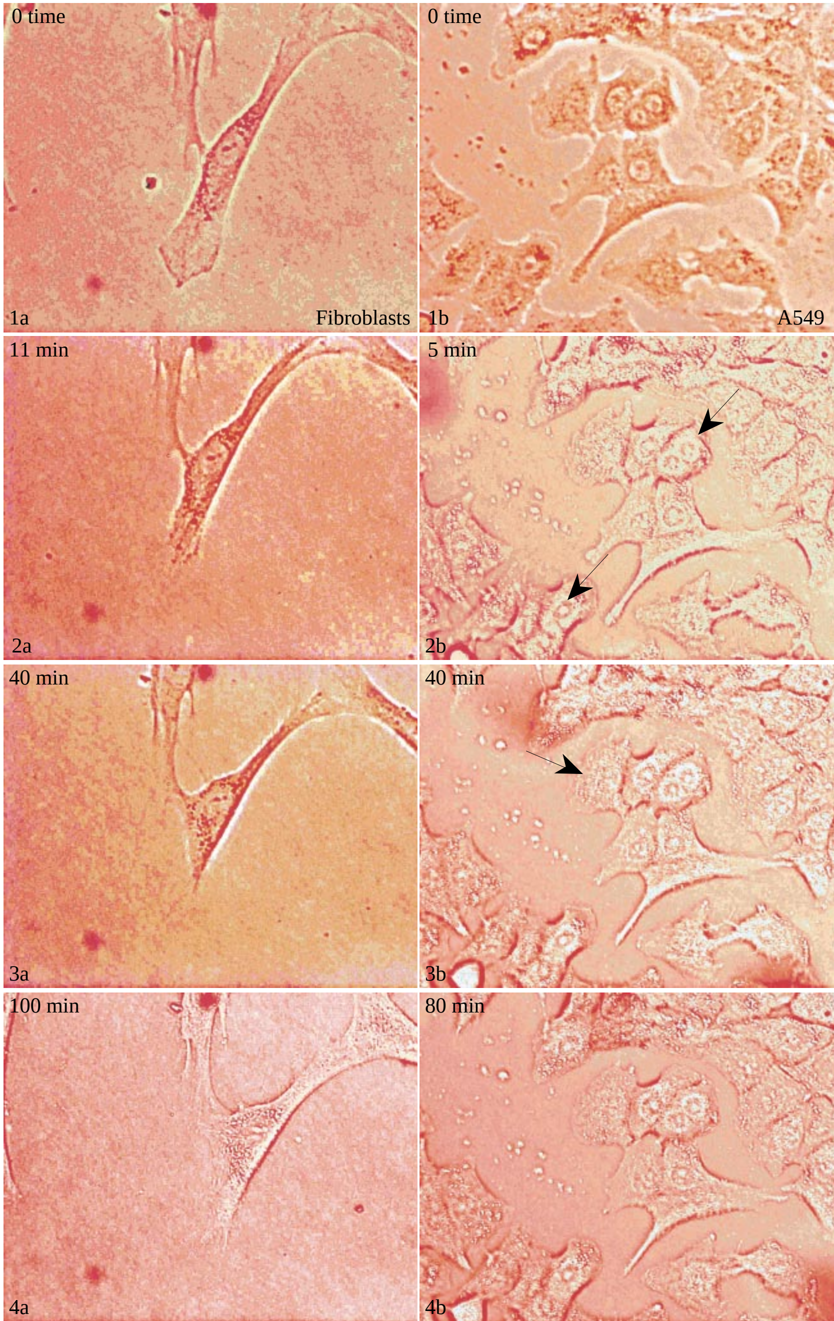


Fig. 10. Normal fibroblasts and cancer cells (A540) grow in MEM media for 24 h, the cells imaged for 10 min in controlled conditions (images 1a and 1b). Images 2a -4a for fibroblasts after changing the media by PM 701. Note that cell retracts in image 2a, but the cell body increased in size (image 3a & 4a). While the cancer cells nuclei attacked by PM 701, indicating by the pale colors surrounded the nuclei (arrow) image 2b). Cells appeared to degenerated (arrow) images 3b & 4b (× 40).

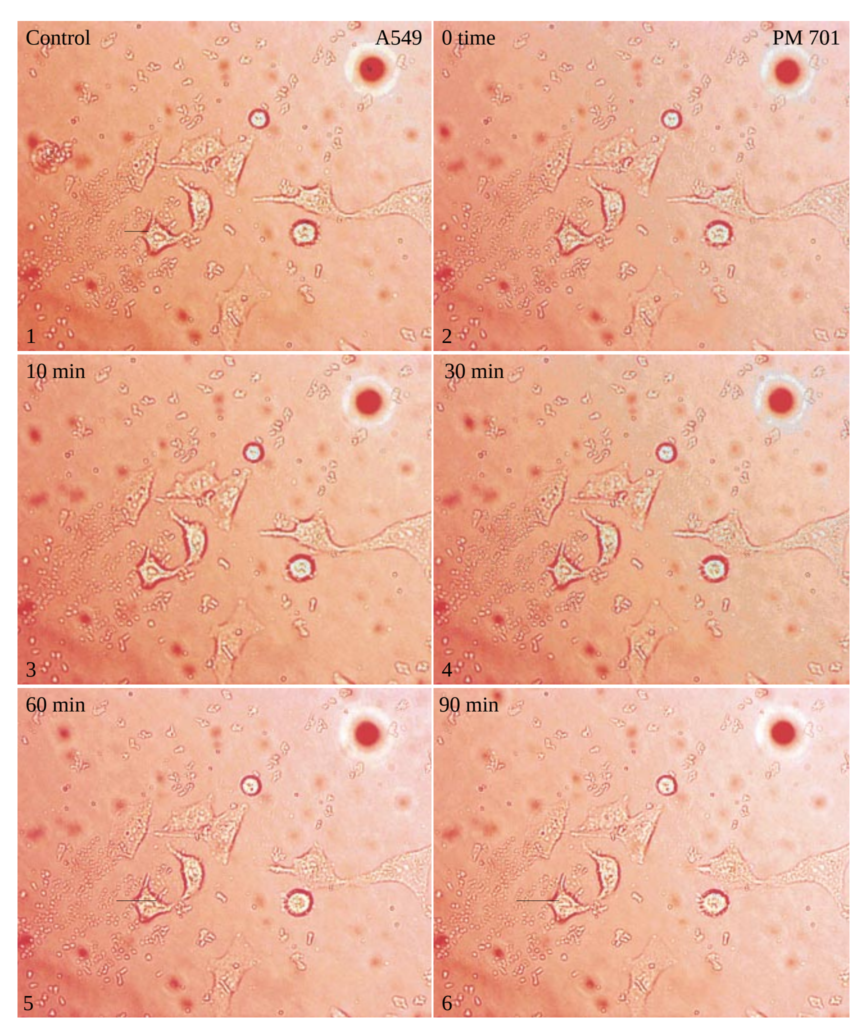


FIG. 11. Live experiment for A540 cells incubated in MEM in the first image, which changed with PM 701 and imaged for timelapse at written time, note the nucleus changing signs lines for same cell.

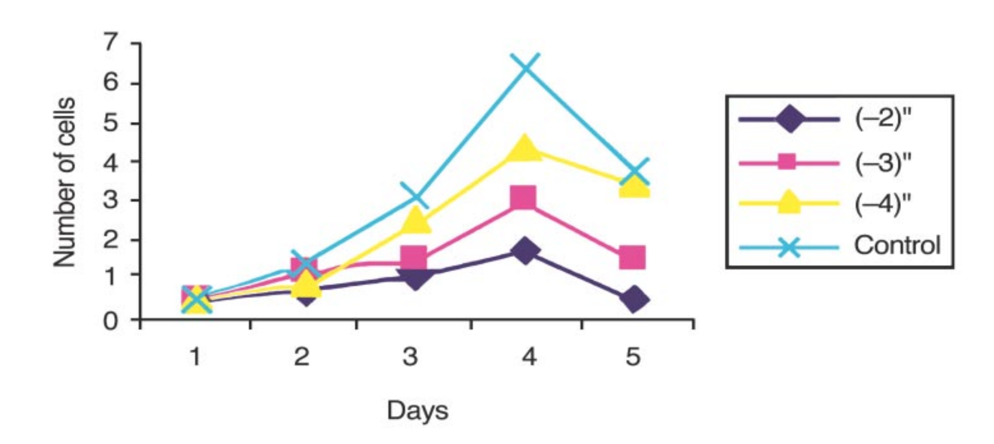


Fig. 12. Curve shows the relationship between cells number and the number of days for incubating cancer cell A549 in different concentration of PM 701 (–1, –2, –3, –4), where the number of cells decreases by increase of the reagent concentration in the incubated medium compared with control cancer cells incubating in MEM.

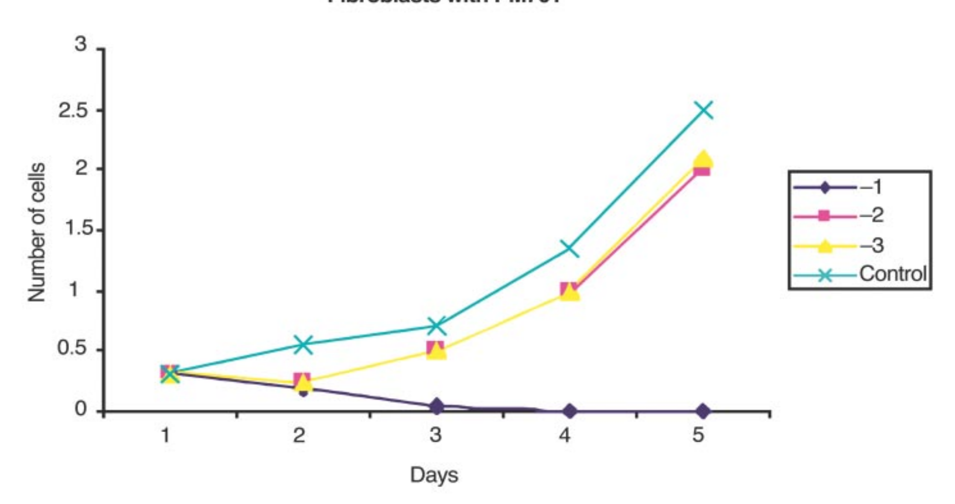


Fig. 13. Curve shows the realtionship between cells and the number of days for incubating normal fibroblasts cells in different concentration of PM 701 (–1, –2, –3). The number of cells appear to have the same progess of the control fibroblasts cells incubating in MEM. Only the curve depress in very high concentration (–1), which may be toxic for the cells.

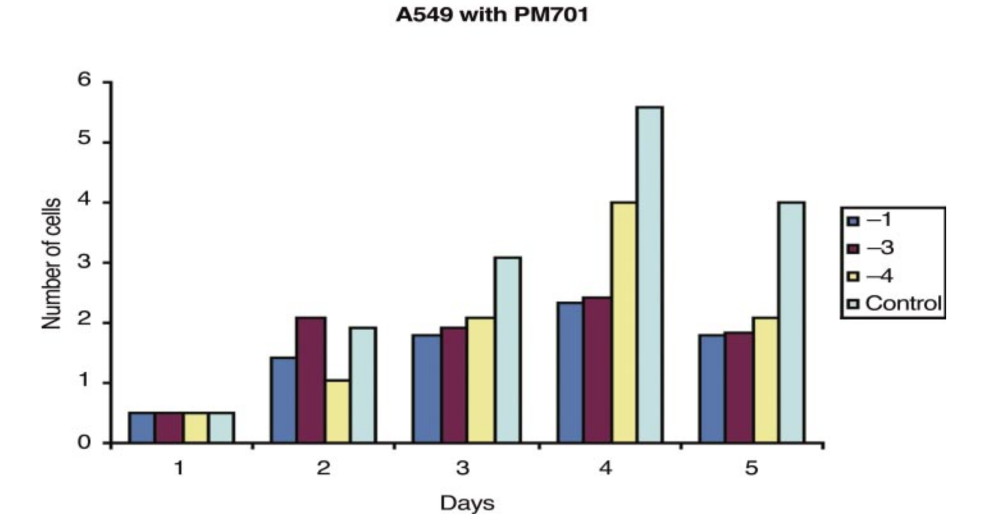


Fig. 14. The diagrams show the same number of cancer cells incubated in PM 701 or MEM as a control for the first 24 h. Whereas, the control cells icrease in number by increase of the number of days, compared with cells incubated in different concentrations of PM 701, see day 4.

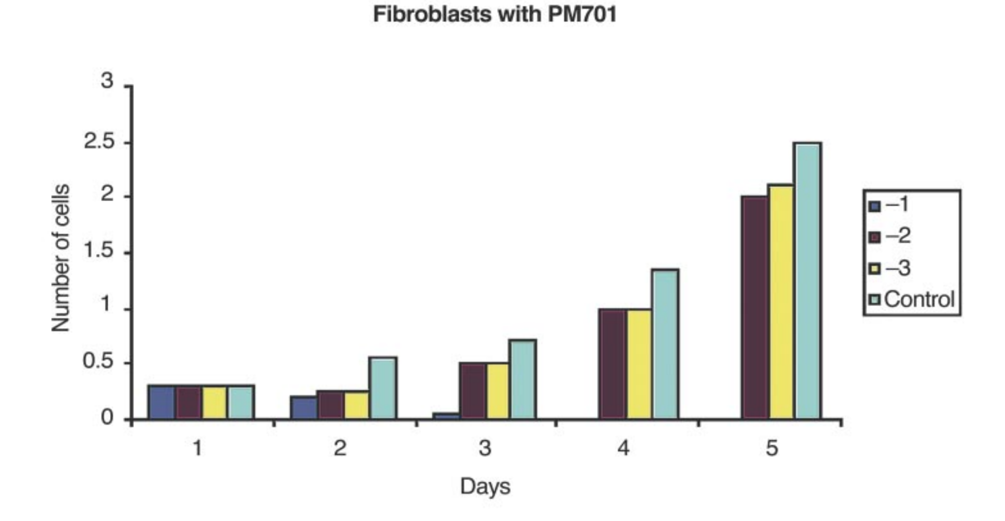


Fig. 15. The diagram indicates that there is no negative effect of different concentration (–2 or –3) of PM 701 on the cell number of fibroblasts comparing with fibroblasts incubating in MEM.

blasts) comfort to the new media after short time and appeared healthy and spread more than in the ordinary media.

Discussion

The achievement in the war against the malignant disease varies greatly, depending on the types of the neoplasm, the stage of the disease and the degree of histological favorably[2]. In spite of the success in satisfactory control of some of the malignant disease and failure in the others with the available modalities, *i.e.*, chemotherapy and radiotherapy[2, 8], we remain far from the ideal treatment, which we can define it as 'the treatment that can selectively kills the malignant cell sparing the normal healthy tissue and the function of the vital organs'. Our study indicated that the PM 701 did fulfill the criteria of the ideal treatment for cancer cells *in vitro* as seen in killing the lung cancer cells, while it has flourishing effect on the normal fibroblast. It is worth mentioning that the effect of this substrate is favorable in low and best in medium concentrations and does not require high concentration to exhibit its best effect as in case of chemotherapy.

Although induction of apoptosis (cell death mediated by caspases) determines responses to cancer therapy, this approach is limited by lack of selectivity in the available apoptosis-inducing agents[8].

The detailed mechanism of action of PM 701 in selective killing of the malignant cells is not known but it is clear, as documented in the live imaging in the process of mixing of the substrate with the culture media that it works on the level of the nuclei. It is clear that the PM 701 isolates the malignant cells from the surrounding, leading to changes in the nucleus, arresting the growth and division of the cells leading to degeneration and death of the cell as seen in the three different experiments with progressive decrease in the number of the cells till the disappearance of the cells in approximately 120 h. On the other hand mixing of the PM 701 with normal fibroblasts show the early precautionary reaction of the cells upon the contact with new substrate by withdrawal of their pseudopodia but with increase in the size of cell body followed in the short time by normal reaction and flourishing (growth & spreading of cells and well developed cytoskeletons and organelles) this flourishing effect was seen in the different concentrations of the media (low and medium), but the high concentration was toxic to the cells, this toxicity can be understood as the cells in the culture media are working independently of the other protective and regulatory mechanism and function of the normal body of intact living organism, so cannot tolerate the concentrated substrate, this is why different concentrations have to be used to test the effect of the substrate avoiding the full or high concentration.

It is clear from the three experiments that this substrate is effective anticancer agent at tissue culture level were it causes cell damage at the nuclear level which in turn leads to arrest of division of the cell and further more degenerative change and death of the cell, these changes were seen to start in the first 10 min. of live experiment which continues leading to disappearance of the cancer cells by 4-5 days.

We are presenting our preliminary results in the use of this substrate (PM 701) *in vitro* level using one type of malignant cells (lung cancer cells A549), which showed reproducible results in selective destruction of the lung cancer cells and flourishing effect on normal skin fibroblasts. The authors will progress to study the effect of PM 701 on other types of cancer cells in future work and animal models to confirm its efficiency as anticancer agent.

Conclusion

In this research, we obtain an anticancer substrate PM 701, which is natural, easily available, cheap, sterile, non toxic and can cause selective cell death of cancer cells and has flourishing effect on normal skin fibroblasts at the tissue culture media. It does not require high concentration as chemotherapy.

These results which were reproducible at the *in vitro* level may lead to successful alternative anticancer agent at *in vivo* level other than radiotherapy or chemotherapy, which destroys the normal as well as cancer cells.

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علاج مثالي يحارب الخلايا السرطانية معمليا:
١ – دراسة على الخلايا المزروعة في المعمل من سرطان الرئة للإنسان

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و [٣] وحدة زراعة أنسجة الخلايا ، مركز الملك فهد للبحوث الطبية

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المستخلص. يعتبر علاج السرطان من أهم المشاكل الطبية المطلوب حلها، حيث لا يوجد في الوقت الحالي علاج مثالي يمكنه القضاء على الخلايا السرطانية دون الإضرار بالخلايا الطبيعية أو التأثير على وظائف الأعضاء الحيوية. و في هذا البحث تم استخدام تقنية زراعة الخلايا لدراسة تاثير المادة (*PM 701*) على نمو خلايا رئة الإنسان السرطانية (*A549*) ومقارنة تأثير هذه المادة على تصرفات الخلايا السرطانية وتأثيرها على الخلايا الطبيعية المزروعة من جلد لإنسان (*human skin fibro-blasts*). وقد أظهرت المادة المدروسة فعالية في القضاء على الخلايا السرطانية للرئة وذلك بتأثيرها على الأنوية ، مما يحد من انقسام الخلايا ويؤدي إلى تحللها وموتها. وفي نفس الوقت أظهرت هذه المادة تأثيراً مغذّيًا على الخلايا الطبيعية المزروعة من جلد الإنسان مما يدل على الأثر العلاجي لهذه المادة بمحاربتها للخلايا السرطانية فقط، ودلت النتائج على تأثير كل التركيزات المستخدمة من هذه المادة مع أفضل تأثير للتركيز المتوسط. كما ظهر التأثير المميت للمادة على الخلايا السرطانية في العشر الدقائق الأولى من التجربة الحية . تم تسجيل الملاحظات والتصوير مباشرة أثناء التجربة. وجدير بالذكر أن هذه التجربة تعتبر الأولى من نوعها لدراسة التأثير المدمر الأمثل للمادة (*PM701*) على الخلايا السرطانية المزروعة في المعمل.